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ORIGINAL ARTICLE

The taxonomic position of the genus *Heydenia* (Pyronemataceae, Pezizales) based on molecular and morphological data

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Abstract Molecular and morphological data indicate that the genus *Heydenia* is closely related to the cleistothecial ascomycete *Orbicula* (Pyronemataceae, Pezizales). Observations on the disposition and the immediate surroundings of immature spores within the spore capsule suggest that the *Heydenia* fruiting bodies are teleomorphs producing early evanescent asci in stipitate cleistothecia. The once advocated identity of *Heydenia* with *Onygena* is refuted on molecular grounds. *Onygena arietina* E. Fischer is transferred to *Heydenia*.

Keywords Ascomycota · Beta tubulin · Cleistothecium · Histology · nuLSU · Orbicula · Phylogeny

Introduction

When a morphologically well differentiated fruiting body has abundant spores but neither asci nor basidia can be seen, one is tempted to assume that it is a conidial fungus. If, in addition, the fruiting body has an unusual form not commonly occurring in ascomycetes or basidiomycetes, the assumption seems confirmed; and if faulty observation or wrong interpretation is used to justify the assumption, it easily becomes accepted as true, without proof, in the mycological literature. One case of such questionable reasoning is exemplified by the genus *Heydenia*.

Since fungi with fruiting bodies of unusual or misleading form are difficult to fit into a classification based on morphology alone, many genera were left unclassified («incertae sedis») or were assigned by guesswork to a taxonomic group until more objective criteria based on analyses of DNA sequences indicated a firm phylogenetic relationship. Examples of such genera are *Torrendia* (stipitate gasteromycete-like, Hallen et al. 2004), *Thaxterogaster* (stipitate sequestrate, Peintner et al. 2002) and *Physalacria* (columnar hollow, Wilson and Desjardin 2005) among the basidiomycetes, and *Neolecta* (columnar, Landvik et al. 2001), *Trichocoma* (cup-shaped with a protruding tuft, Berbee et al. 1995) and *Heydenia* (stipitate cleistothecium, this paper) among the ascomycetes.

The genus *Heydenia* with a single species, *H. alpina*, was proposed by Fresenius (1852) for a small fungus growing on plant debris and mosses in the Swiss Alps. This species is characterised by a dark, loosely stuffed or hollow stipe with a pseudoparenchymatous wall flaring out on top to support a pale mass composed of hyaline spores and radiating hyphae. The spores were described as being produced in chains on verruculose hyphae and were reported by Saccardo (1886) as «conidia pleurogena, typice catenulata». This inspired the opinion that *H. alpina* is a conidial anamorph of unknown taxonomic position, presumably within the Ascomycota (Kirk et al. 2008).

The *Heydenia* fruiting body was called synnema-like, coremium-like or a sporodochium by various authors (e.g. Samson and Hintikka 1974; Heim 1934; Saccardo 1882), but we agree with Cavara (1903) who rejected such comparisons because of the presence of pseudoparenchymatous contexts in *Heydenia*.

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Two recent collections belonging to two different *Heydenia* species from the Swiss Alps enabled us to establish cultures and to use DNA sequences of the ribosomal large subunit (LSU) to study the taxonomic position of these fungi. One collection agreed with the original description of *H. alpina* Fres., but the second collection did not fit any described *Heydenia* species. Instead, it is identical to a fungus called *Onygena arietina* by Fischer (1897, 1920). This fungus is therefore transferred to the genus *Heydenia*.

The present paper describes the anatomy of the fruiting bodies of *Heydenia* and reports on the taxonomic position of this genus based on DNA sequence data.

Materials and methods

Species and isolates

Heydenia alpina Fresenius. **Switzerland**. Ct. VS, Furka pass near Oberwald, at about 2000 m, on soil under subalpine bush vegetation, 06 Aug. 2007, leg. B. Senn-Irlet and H.U. Aeberhard (ZT Myc 3556). Culture: isolate 0732, CBS 129149. Additional collections studied (morphology only): **Switzerland**. Ct. TI, Bedretto, Val Corno, Pian Tondo, at 2250 m, on soil between mosses and dead plant remains (*Salix*), 26. Aug. 1988, leg. E. Horak 4101 (ZT). **Switzerland**. Ct. GR, Scharl, at 2315 m, on soil and dead plant material (*Dryas, Calluna*), 01 Sept. 1988, leg. E. Horak 4135 (ZT). **France**. Savoie, Bourg-St. Maurice, Lac Marloup, at 2600 m, on dead petioles of *Cirsium spinosissimum* (photograph published in Hairaud and Moreau 2002), 26 June 2000, leg. M. Hairaud (comm. P. Moreau 62601).

Heydenia sp.. **Switzerland**. Ct. GR, Vnà - Ramosch, Piz Arina, at 2680 m, on naked silicate rock, 24 June 2005, leg. S. and R. Stegmann [LAU (HC 05/012) and ZT (Myc 3557)]. Culture: isolate 0701, CBS 129150. Additional collections studied (morphology only): **Switzerland**. Ct. VD, Anzeindaz, Hauts Crots, at 2100 m, on naked lime stone, early June 1976, leg. P. Clavel (as *Heydenia* sp.) (ZT). **Switzerland**. Ct. GR, Arosa, Sandboden, at 2050 m, on *Calluna vulgaris*, 17 May 1971, leg. E. Rahm (as *H. alpina*) (ZT). **Switzerland**. Ct. GR, Bernina, 1971, leg. J. Dingley (as *H. alpina*) (ZT). **France**: Pic du Midi de Bigorre, leg. Roux, 1970 (PC 0093189; Beller 800 as *H. alpina*).

Orbicula parietina (Schrad.) Hughes. **Canada**. Ontario, York Co., Nashville, "on decaying clover hay", 25 Apr. 1959, leg. R. F. Cain, University of Toronto Cryptogamic Herbarium 34267 (part of collection deposited in ZT). – *Anixia truncigena* Hoffm., Herbier Fuckel 1894, Fungi rhenani 1070, "Ad lignum abietinum putridum", leg. K. Fuckel, annotated "= *Orbicula parietina*" (ZT).

Cultures

The two recent *Heydenia* collections were still living when they arrived in our laboratories and could be isolated in pure cultures from fruiting bodies of specimens 20 months (*Heydenia* sp., isolate 0701) or 1 month (*H. alpina*, isolate 0732) after collection. Spores were dispersed on potato-dextrose-agar (PDA) dishes supplemented with the antibiotic oxytetracycline, on which approximately 90% of the spores germinated within 24 h. Single spores were then picked at 160×magnification with the aid of a micromanipulator and grown up on a new dish into separate colonies.

DNA extraction and sequencing

Genomic DNA was extracted from mycelia of subcultures of single spore isolates grown atop cellophane disks on PDA plates, or directly from fruiting bodies. DNA was prepared from freeze dried mycelia following a CTAB-SEVAG method as outlined previously (Brem and Leuchtmann 2003), or from fruiting bodies using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Total DNA of samples were quantified by comparing bands to known standards of Lambda DNA (Promega) on a 1% agarose gel.

The 18S large subunit nuclear ribosomal RNA gene (nuLSU) was amplified using primers LROR and LR5 (Moncalvo et al. 2000), and, in addition, for sequencing the internal primer LR3R (Moncalvo et al. 2000). Amplification of intron rich portions of beta-tubulin (*tubB*) were performed using primer pairs Bt1a and Bt1b (approximate size of PCR product 540 bp) and Bt2a and Bt2b (408 bp) (Glass and Donaldson 1995). The nuclear ribosomal RNA 5.8S gene and the two internal transcribed spacers (ITS1 and ITS2) were amplified using primers ITS1, ITS3 and ITS4 (White et al. 1990). Reactions were performed in 25 µl volumes containing 5 µl 5X GoTaq Flexi buffer (Promega, Madison, WI, USA), 2.5 mM $MgCl_2$, 1,25 µl dNTPs (2.5 mM), 1.0 µl of each primer (10 mM),1.25 U GoTaq Flexi DNA polymerase (Promega), and 10 ng DNA template. Reactions were carried out in a GeneAmp PCR System 9700 thermocycler (Applied Biosystems, Foster City, CA, USA) programmed for an initial incubation of 3 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 52°C, and 3 min at 72°C; then a final extension of 7 min at 72°C. PCR products and a 100 bp ladder as size standard were separated by electrophoresis in 1.5 % agarose gel in 0.5 % TBE, and visualized by SYBR® Green I (Molecular Probes, Eugene, OR, USA) under UV light.

For purification PCR products were treated with 1 µl exonuclease I and 2 µl shrimp alkaline phosphatase (per 10 µl product) and incubated at 37°C for 15 min and 80°C for 15 min, respectively, then cleaned with Sephadex.

Sequencing reactions were performed in 10 µl volumes using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Austin, TX, USA) with PCR conditions as recommended by the manufacturer. Both strands of the purified products were sequenced using the primers indicated above. Sequence products were separated on a capillary 3130xl Genetic Analyzer (PE Applied Biosystems), then sequences edited and assembled using Sequencher 4.6 (GeneCodes Corp., Ann Arbor, MI, USA). The new sequences have been deposited in GenBank (National Center for Biotechnology Information, Bethesda, Maryland, USA; http://www.ncbi.nlm.nih.gov/): nuLSU of *Heydenia* sp. isolate 0701 (accession no. HQ596525) and *H. alpina* isolate 0732 (HQ596526); tubB of *Heydenia* sp. (HQ688650) and *H. alpina* (HQ688651); ITS1-5.8S-ITS2 of *Heydenia* sp. (HQ688652) and *H. alpina* (HQ688653).

Phylogenetic analyses

Analysis of nuLSU rDNA sequences included 42 reference sequences published by Perry et al. (2007) and three outgroup sequences that were downloaded from GenBank (accession numbers shown on the tree in Fig. 1). New and downloaded sequences were aligned with Clustal X (Thompson et al. 1997) and manually adjusted using MacClade 4 (Maddison and Maddison 2005). Because of ambiguous alignments of the sequences at several regions, a total of 68 nucleotides were excluded from phylogenetic analyses. The aligned dataset is available via TreeBASE [http://purl.org/phylo/treebase/phylows/study/TB2:S11244].

Maximum parsimony (MP) analyses were performed by heuristic search in PAUP* 4.0b10 (Swofford 2003). Character changes were unweighted and unordered with gaps treated as missing data. Trees were built by 100 iterations of random stepwise taxon addition using tree bisection-reconnection (TBR) branch swapping. Maximum likelihood (ML) trees were identified in PAUP* 4.0b10, using a general time reversible (GTR) model and maximum likelihood settings from best-fit model selected by hierarchical likelihood ratio tests in MODELTEST 3.6 (Posada and Crandall 1998). Searches with the nuLSU dataset used a GTR+G+I model of sequence evolution (proportion of invariable sites 0.5, gamma distribution shape 0.6167). Trees were inferred from 100 iterations with random taxon addition order and different number of seed. Confidence levels of individual clades were assessed by MP bootstrap analyses (Swofford et al. 1996) using 1000 heuristic replicates. Bayesian posterior probabilities of nodes were calculated with MrBayes version 3.1.2 (Ronquist and Huelsenbeck 2003) using a GTR+I+G model as determined above. Searches were run for 1'000'000 generations with sampling one out of every 100 trees. The first 2500 trees were discarded as 'burn-in', while the remaining trees were used to determine posterior probabilities of clades. The heating scheme used the default settings in MrBayes.

Portions of *tubB* sequences (from primers Bt2) and six reference sequences of *Neonectria* and *Fusarium* downloaded from GenBank were aligned and analysed as above. Maximum likelihood settings used a GTR+G model with invariable sites none and gamma distribution shape 0.228.

Fungal morphology

Dry but still living collections of *H. alpina* and *Heydenia* sp., and dry material of *Orbicula parietina* from herbaria were rehydrated in a wet chamber at room temperature for 12–36 h or in a 4–5 % ammonia solution for 6–12 h. Small rehydrated samples were squashed for light microscopy in glycerol - methyl cellosolve - sodium hydroxide (GMS), SDS Congo red, toluidine blue O, cotton blue, patent blue V, Sudan III and in Baral's and Melzer's iodine solutions (Clémençon 2009). Larger samples or whole rehydrated fruiting bodies were fixed with aldehydes, dehydrated with methyl cellosolve, carried through ethanol, propanol and butanol and embedded in a 65:35 glycol methacrylate - butyl methacrylate mixture (Clémençon 2009). Microtome sections were stained with toluidine blue, iron chloride - haematoxylin, and tannin - iron chloride - haematoxylin (Clémençon 2009). Preparations were photographed with an Olympus DP11 digital camera mounted on a Leitz Orthoplan microscope equipped with NPL Fluotar and PL Apochromatic lenses. The photographs were processed with Adobe Photoshop CS4 Extended on a Macintosh computer (reframing, contrast adjustment, background cleaning, moderate sharpening or photomerging).

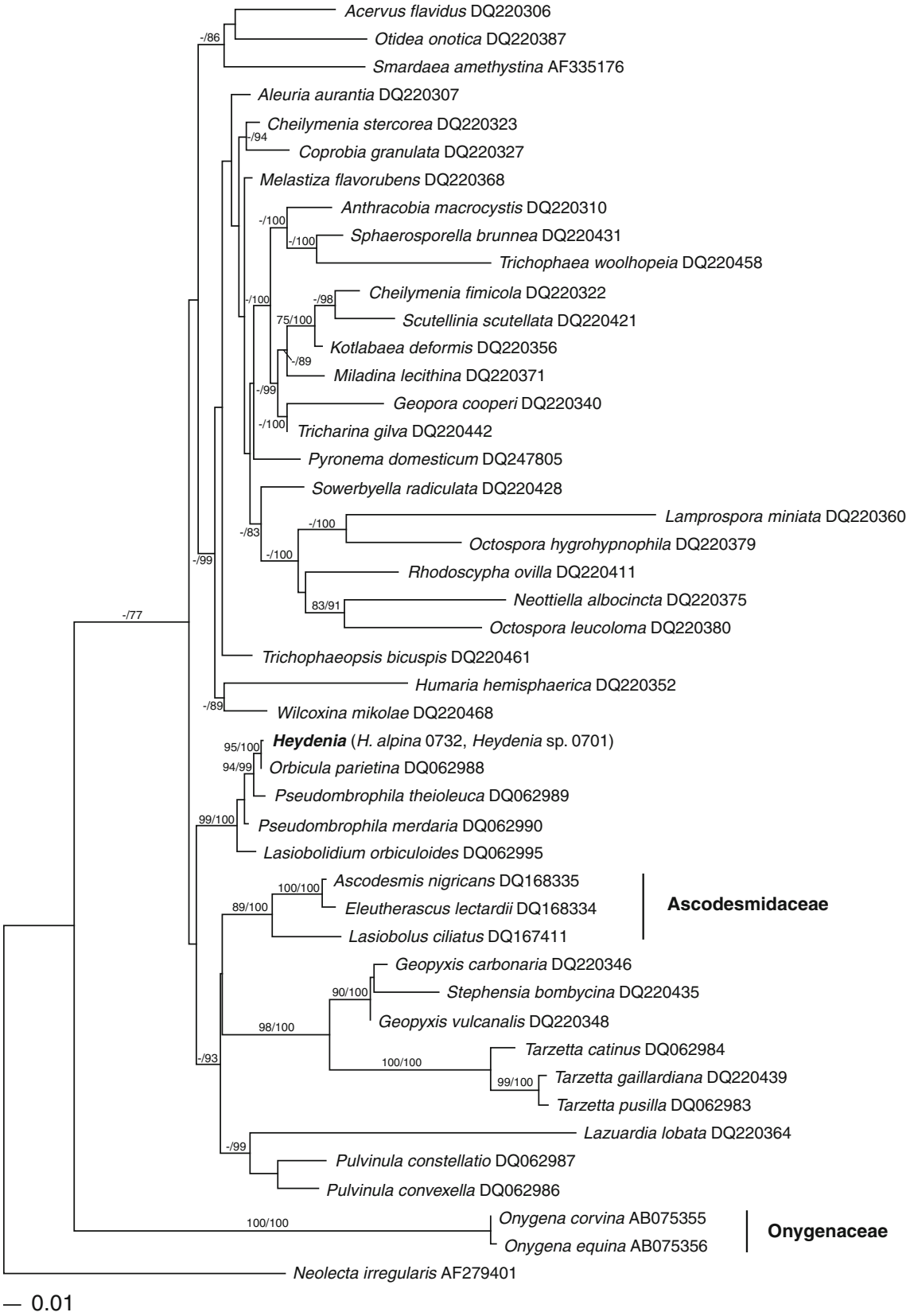
Results

Cultures

The colonies grew well in PDA Petri dishes at room temperature (20–25°C) forming a white, cottony mycelium, but refused to produce fruiting bodies. Attempts to induce fruiting by exposing the cultures to UV light, frost or sunlight, as well as the addition of sterilised plant debris or naked stones, injuring the growing mycelium with a sterile scalpel, inverting the Petri dishes and the use of depletion cultures (Clémençon 2009), all failed.

Phylogenetic analyses

Sequences of ITS1–5.8S–ITS2 and nuLSU genes of *Heydenia* sp. (isolate 0701) and *H. alpina* (isolate 0732) were identical. Also, results did not differ when using DNA


Acervus flavidus DQ220306
Otidea onotica DQ220387
Smardaea amethystina AF335176
Aleuria aurantia DQ220307
Cheilymenia stercorea DQ220323
Coprobia granulata DQ220327
Melastiza flavorubens DQ220368
Anthracobia macrocystis DQ220310
Sphaerosporella brunnea DQ220431
Trichophaea woolhopeia DQ220458
Cheilymenia fimicola DQ220322
Scutellinia scutellata DQ220421
Kotlabaea deformis DQ220356
Miladina lecithina DQ220371
Geopora cooperi DQ220340
Tricharina gilva DQ220442
Pyronema domesticum DQ247805
Sowerbyella radiculata DQ220428
Lamprospora miniata DQ220360
Octospora hygrohypnophila DQ220379
Rhodoscypha ovilla DQ220411
Neottiella albocincta DQ220375
Octospora leucoloma DQ220380
Trichophaeopsis bicuspis DQ220461
Humaria hemisphaerica DQ220352
Wilcoxina mikolae DQ220468
Heydenia (H. alpina 0732, Heydenia sp. 0701)
Orbicula parietina DQ062988
Pseudombrophila theioleuca DQ062989
Pseudombrophila merdaria DQ062990
Lasiobolidium orbiculoides DQ062995
Ascodesmis nigricans DQ168335
Eleutherascus lectardii DQ168334
Lasiobolus ciliatus DQ167411
Ascodesmidaceae
Geopyxis carbonaria DQ220346
Stephensia bombycina DQ220435
Geopyxis vulcanalis DQ220348
Tarzetta catinus DQ062984
Tarzetta gaillardiana DQ220439
Tarzetta pusilla DQ062983
Lazuardia lobata DQ220364
Pulvinula constellatio DQ062987
Pulvinula convexella DQ062986
Onygena corvina AB075355
Onygena equina AB075356
Onygenaceae
Neolecta irregularis AF279401
0.01

◀ **Fig. 1** Maximum-likelihood (ML) tree of representative species of Pyronemataceae and Ascodesmidaceae (with GeneBank accession numbers) including *Heydenia* spp. based on nuLSU sequence data. MP bootstrap percentages and Bayesian posterior probabilities greater than 70% are indicated on the branches (− designates a value lower than 70%). The tree includes sequences of two *Onygena* species and is rooted with the outgroup taxon *Neolecta irregularis*

from fruiting bodies or from cultured mycelia. Maximum likelihood (ML) analyses of nuLSU sequences placed the two *Heydenia* species close to the cleistothecial *Orbicula parietina* and apothecial *Pseudombrophila* spp., together with the cleistothecial *Lasiobolidium orbiculoides* in a well-supported clade (99/100 %) within the Pyronemataceae (Fig. 1). Next to this clade was a divers clade containing representative species of the Ascodesmidaceae, globose-spored species of *Pulvinula* and species of *Geopyxis*, *Lazuardia*, *Stephensia* and *Tarzetta*. Species of *Onygena* (Onygenaceae), with which *Heydenia* once was confused, were placed outside and very distant of the members of the Pyronemataceae. Parsimony analyses (MP) of nuLSU sequences resulted in 12 most parsimonious trees that displayed similar topology of major clades to those obtained by ML analyses (data not shown).

The two *Heydenia* species could be clearly distinguished based on *tubB* sequences. They differed in seven single nucleotides out of a 360 bp portion of the *tubB* gene (from primers Bt2 covering one intron). Polymorphisms of this magnitude in *tubB* sequences are comparable to those found between species pairs of unrelated *Neonectria* and *Fusarium* (Fig. 2) supporting distinctness of the two *Heydenia* isolates at the species level. In a second portion of *tubB* (from primers Bt1) differences between *Heydenia* species affected eight nucleotide sites plus a 3 bp indel (data not shown).

Morphology of the *Heydenia* fruiting body

The following descriptions are based on an analysis of microtome sections of *H. alpina* and *Heydenia* sp. fruiting bodies, which are composed of an almost hollow stipe supporting an ellipsoid to globular, apical capsule filled with a gleba consisting of spores and a few radiating hyphae. The stipe is conical or cylindrical, open at its base

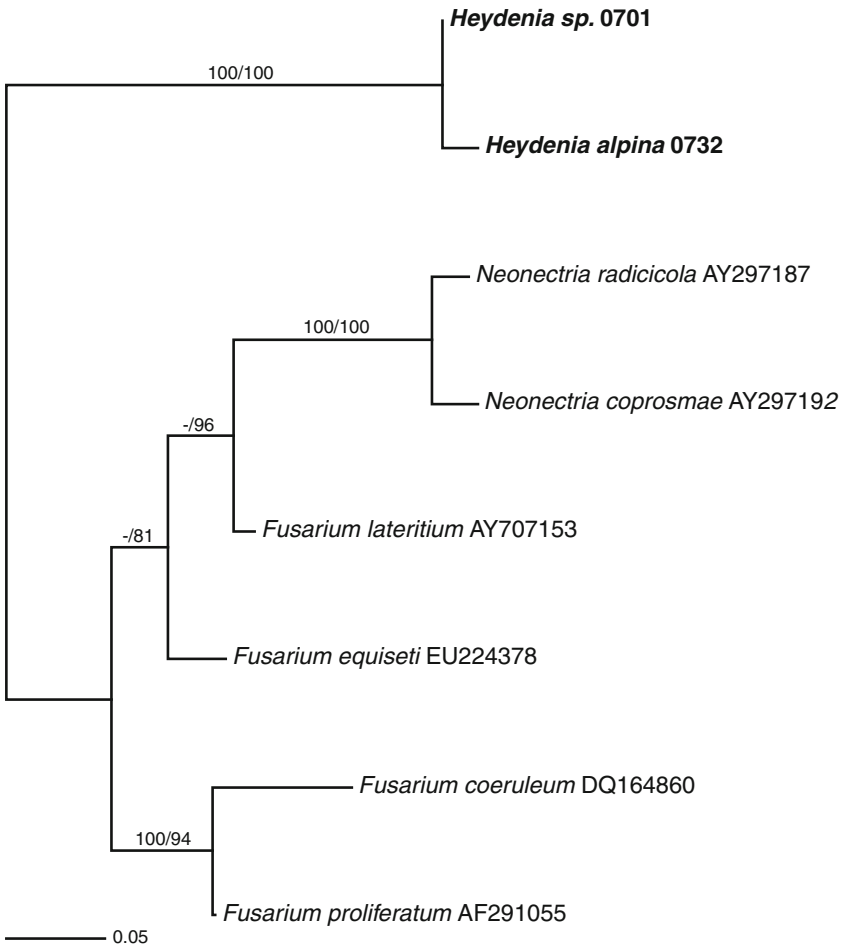


Fig. 2 Maximum-likelihood tree of *Heydenia* spp. and selected outgroup taxa of *Neonectria* and *Fusarium* based on a 408 bp portion of *tubB* gene sequences (from primers Bt2). MP bootstrap percentages and Bayesian posterior probabilities greater than 70% are indicated on the branches (− designates a value lower than 70%)

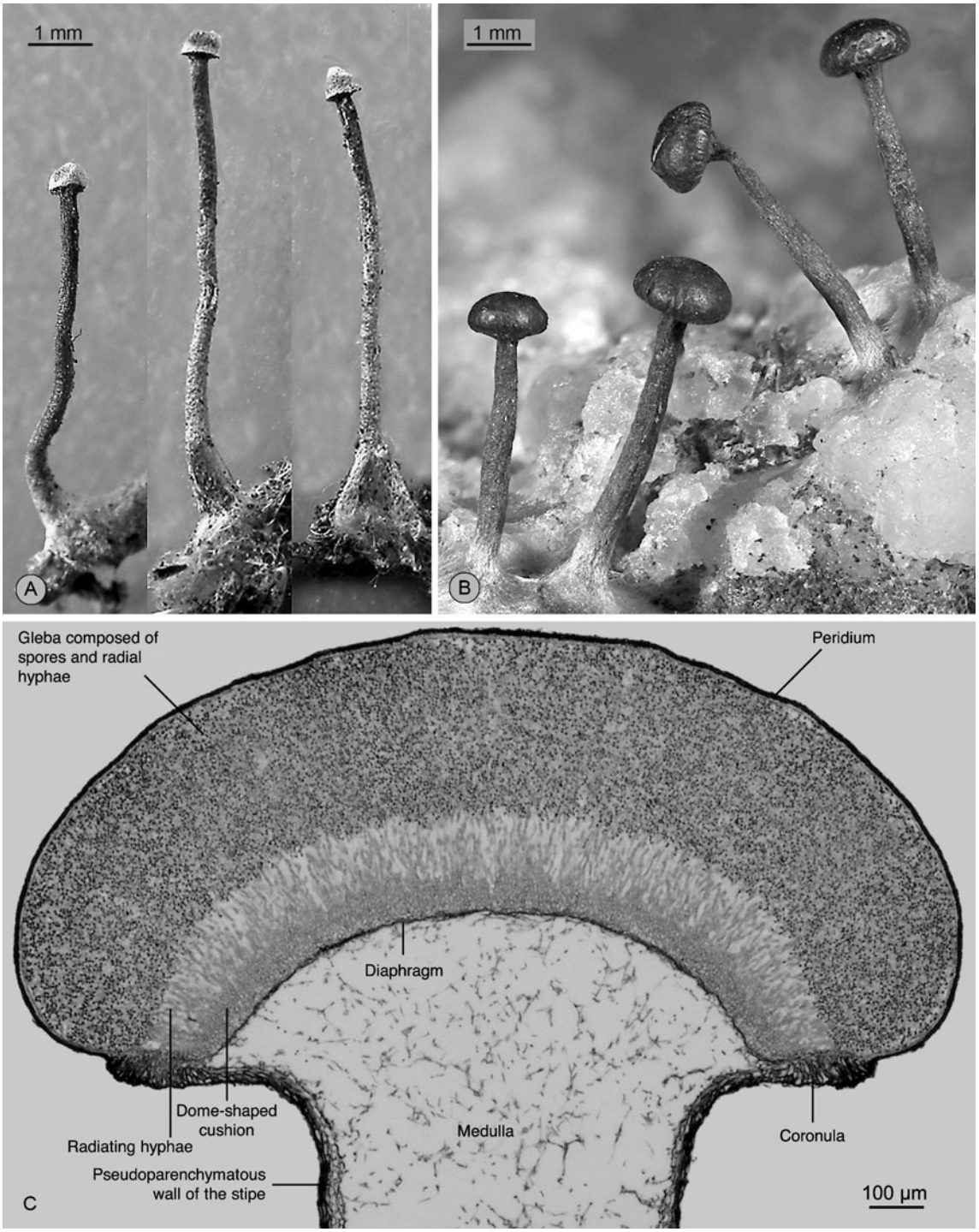


Fig. 3 Fruiting bodies of the two *Heydenia* species studied. **a** *H. alpina*, growing on dead plant material, spore mass naked; leg. E. Horak 4135 (ZT). **b** *H. arietina*, growing on naked siliceous rock, peridium still present; leg. S. and R. Stegmann HC 05/012 (LAU), photograph Erich Zimmermann. **c** Longitudinal section through a mature stipitate fruiting body of *H. arietina* with the peridium still in place, identification of the parts (Stegmann HC 05/012)

but covered with a white to pale brown, dense mycelium growing onto the substrate (plant debris or naked rock) and fixing the fruiting body to it (Fig. 3a, b). The basal mycelium is a weft of colourless or very pale beige, thick-walled, septate hyphae that originate from cells in the outer layer of the stipe wall.

The capsule is supported by a more or less horizontal widening of the stipe wall, the coronula (Fig. 3c). It is

thicker than the stipe wall, and its outer rim connects to the peridium of the capsule. Inside, the basal part of the capsule is occupied by a dome-shaped, pseudoparenchymatous cushion (Fig. 3c). The space beneath the dome-shaped context is continuous with the space in the stipe, and both contain a loose, tenuous medulla. In young fruiting bodies the medulla intergrades into the dome-shaped pseudoparenchymatous context, but in fully mature specimens of *Heydenia* sp. part of the medulla is collapsed into a thin, dark brown layer called the diaphragm supporting the dome-shaped pseudoparenchymatous cushion (Fig. 3c, 4a). The fragile peridium soon breaks away to expose the dry, whitish to pale beige mass of spores and radiating hyphae. In *Heydenia* sp. a dehiscence furrow is located at the rim of the coronula already mentioned by Fischer (1897, 1920).

The radiating hyphae of the gleba originate in the dome-shaped pseudoparenchymatous cushion. Their basal parts are moniliform, but the upper parts are narrowly cylindrical and penetrate into the spore mass. Towards the peridium the hyphae deviate more and more from their radiating orientation and finally become about parallel to the peridial wall. The cells of the radiating hyphae are multinucleate.

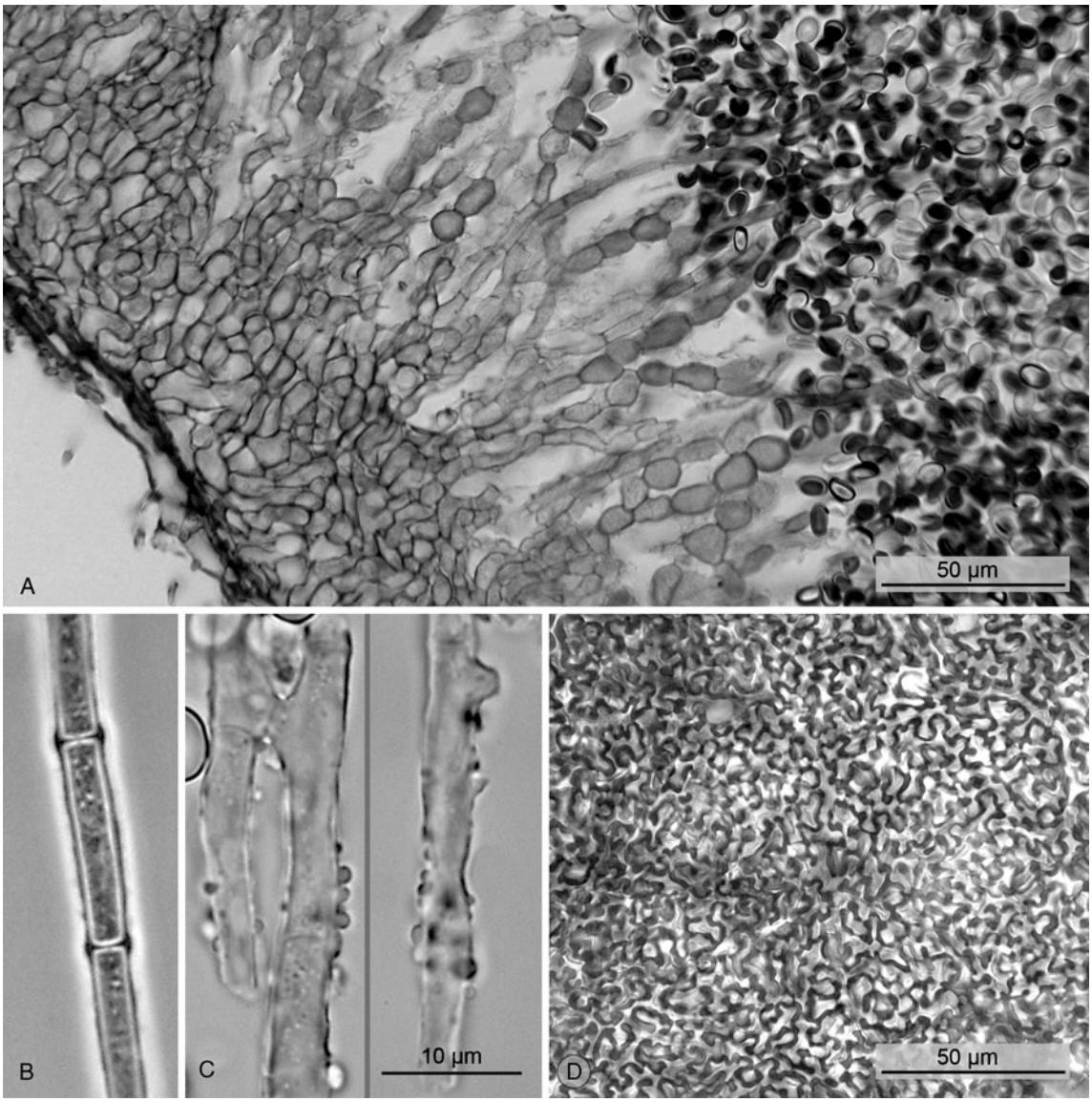


Fig. 4 Anatomy of the *Heydenia* fruiting body. **a** Diaphragm, pseudoparenchymatic dome-shaped context, and radiating hyphae with moniliform basal part and cylindrical apical part penetrating between the spores. The diaphragm consists of compressed medullar hyphae (lower left). **b** Upper part of a radiating hypha from the gleba with thick walls and with bulging rings located over the septa. Phase contrast (scale as in Fig. 4c). **c** The lower part of the radiating hyphae frequently bear resin-like droplets and incrustations. H-connections are frequent but easily break in squash mounts (upper right). **d** The outer layer of the peridium consists of puzzle-like cells with thick brown walls. – **a**, **b**, **d**. *H. arietina* leg. Stegmann; **c**. *H. alpina* leg. Moreau

They anastomose frequently, both in the moniliform and in the cylindrical parts. The septa are often covered with a narrow but slightly bulging mass that gives the hyphae a characteristic appearance (Fig. 4b). The walls are sometimes incrusted with small wart-like solid droplets, especially in the moniliform part (Fig. 4c). Except for these incrustations, the walls of the radiating hyphae are smooth and do not show any sign of warts or pedicels from spore formation, contrary to the report by Fresenius (1852) and Heim (1934), but in agreement with Fischer (1920) and Rytz (1923).

The peridium of the spore capsule consists of two layers. The outer layer is dark brown or almost black and made from strongly melanised, thick-walled hyphal cells arranged in a pseudoparenchymatous way (Fig. 4d). The inner layer is similar but the cells are thin-walled and only slightly pigmented.

The pseudoparenchymatous wall of the stipe consists of an outer cortex with thick-walled, strongly melanised cells and an inner layer of thin-walled, less melanised or even colourless cells. Young stipes are stuffed with a conspicuous medulla, but in fully mature ones the medulla is much reduced, as already noticed by Fischer (1897, 1920). The stipe stretches during maturation of the fruiting body by elongation of the cells of the outer layer and by increase of the volume of the cells of the inner layer.

The coronula is composed of radially elongate cells, as already described and illustrated by Fischer (1920). In *Heydenia* sp. it is at first steeply slanted and becomes about horizontal in mature cleistothecia, but the developmental morphology of the *H. alpina* coronula is unknown. In cross section, the coronula of *Heydenia* sp. is cushion-like, that of *H. alpina* is triangular, as already noticed by Fresenius (1852).

The spores are almost spherical to clearly ellipsoid, moderately thick-walled, colourless, smooth and devoid of a germ pore or germ slit. No bud scar or any other remains are present that could indicate a blastic mode of spore formation. They are not connected by minute intermediate cells, contrary to the report by Fresenius (1852). The wall appears homogeneous in water or alkaline solutions, but toluidine blue reveals two wall layers. In cotton blue (dissolved in concentrated lactic acid) the spore wall usually remains unstained even after heating, but in some spores the outer layer becomes blue. Toluidine blue stains slowly. After about 15 min. the outer spore wall layer becomes pale lilac, and after prolonged staining it appears blue to dark grey-blue. The inner wall layer remains unstained. Congo red does not stain, and the wall is inamyloid in Melzer's solution.

Complete asci and alive ascogenous hyphae with cytoplasmic content were not seen, but in the spore capsule of younger fruiting bodies many narrow, thin-walled and empty hyphae are present in the spaces between the moniliform parts of the radiating hyphae. They escape observation in squash mounts of the spore mass and have not been reported by earlier investigators, but they are clearly visible in properly stained microtome sections and in unstained sections observed with phase contrast microscopy. At first they almost completely fill the available space (Fig. 5a), but in older fruiting bodies they become more and more sparse and finally even rare (Fig. 5b). In the lower part of the spore mass they can be seen to penetrate between the spores, but soon they become almost impossible to trace any further.

Inside the spore capsule of fully mature fruiting bodies the spores are not arranged in any recognisable order and no indication of the mechanism of spore formation can be detected; no trace of any asci is visible, as already noted by Fischer (1897, 1920) and Rytz (1923). But in younger fruiting bodies eight immature spores often line up in linear groups (Fig. 5c-f). Many of these groups are naked and can be recognised only by their immature aspect contrasting with the mature spores of the immediate neighbourhood. Sometimes adjacent young spores are laterally connected by very fine wall fragments clearly different from the walls of the radiating hyphae (Fig. 5e). Close examination of the old permanent slides made by E. Fischer revealed a similar column of eight spores still enclosed in a barely visible ascus wall in one hand made section (Fig. 5f). In squash mounts of the spore mass, numerous small fragments become visible after staining or in phase contrast, but it is impossible to trace their origin to asci. In microtome sections, however, the wall fragments remain in place and can be seen in phase contrast. It is, therefore, not amazing that they went unnoticed by earlier investigators who never used microtomy in their studies.

Taxonomy

Heydenia Fresenius emend. Leuchtm. & H. Clémençon

Ascomata composed of an almost hollow stipe supporting an ellipsoid to globular, apical cleistothecium filled with a gleba. The peridium consists of two pseudoparenchymatous layers. Within the gleba elongate asci with eight ascospores are produced. The asci disintegrate at an early stage to leave a mass of ascopores and a few radiating hyphae. The ascospores are spherical to ellipsoid, moderately thick-walled, colourless, smooth and devoid of a germ pore or germ slit.

Type species H. alpina Fresenius

Heydenia arietina (E. Fischer) Leuchtm. & H. Clémençon, comb. nov.

MycoBank MB 561058

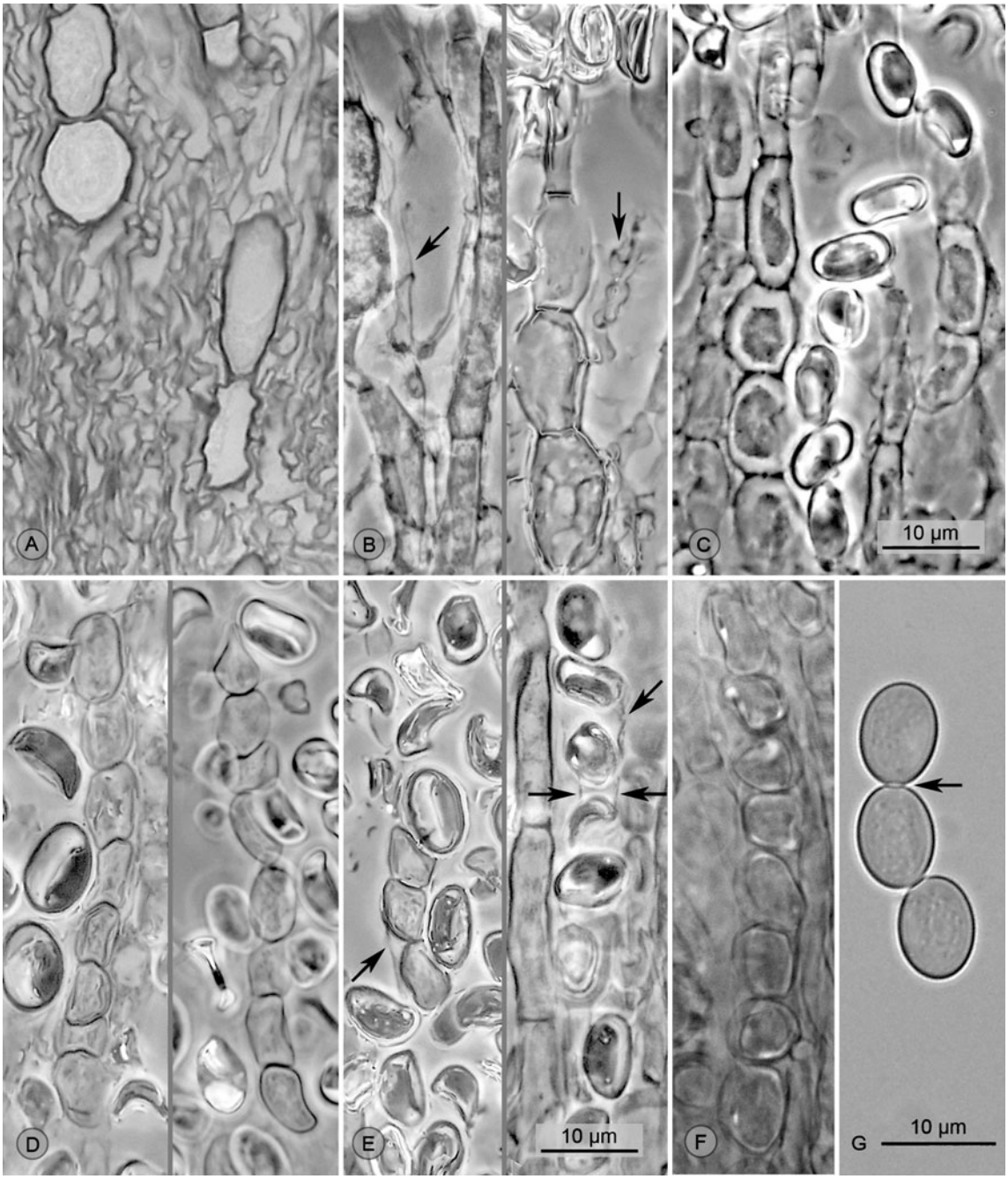


Fig. 5 Presumed ascogenous system in an immature fruiting body of *Heydenia arietina.* **a** Thin, densely packed, thin-walled and empty hyphae fill the space between the moniliform part of the radiating hyphae (the large structures) below the gleba. These hyphae decay and are not present in mature fruiting bodies (same scale as in Fig. 5c). **b** Just below the gleba, where the radiating hyphae become cylindrical, remains of thin-walled, empty hyphae are sometimes visible (same scale as in Fig. 5c). **c** Between the moniliform hyphae well below the gleba columns of eight spores suggest their origin in isolated asci whose walls have already disappeared. The topmost two spores have been slightly dislodged. This photograph shows the morphological difference between the spores and the moniliform cells of the radiating hyphae. **d** Two columns of eight immature spores located in the gleba and surrounded by mature spores (same scale as in Fig. 5e). **e** Two columns of immature spores with fragments of the ascus wall still in place (arrows). **f** A column of eight spores in a permanent slide made by E. Fischer (same scale as in Fig. 5e). **g** Where Becke lines (the bright haloes around the spores) intersect, they may produce the illusion of a tiny intermediate cell between two spores. – **a**, **b**, **c**, **d**, **e**. **France**: leg. Roux

Basionym Onygena arietina E. Fischer, in Rabenhorst's Kryptogamen-Flora, Zweite Auflage, Erster Band: Die Pilze. V. Abtheilung, p. 106. 1897.

Typification No type was designated by E. Fischer, but permanent slides made by the author from the original material are preserved at the Institute of Plant Sciences,

University of Bern, Switzerland. Part of this material is chosen for lectotypification: Switzerland, Davos, on the horn of a living ram (indicated by Fischer 1897, 1920), 09 May 1893, leg. J. Amann. Eight longitudinal sections from a single fruiting body (the designated lectotype), made by E. Fischer were mounted in two original permanent slides. One partly deteriorated slide is left untouched, the other one was used to make three new permanent slides, resulting in a total of four slides that are all part of the lectotype. They are now deposited at the Herbarium of ETH Zürich (ZT).

Discussion

Taxonomic position of the genus *Heydenia*

Phylogenetic analysis of nuLSU sequences clearly established the cleistothecial genus *Heydenia* as a member of the Pyronemataceae. *Heydenia* spp. are placed in a well-supported clade that has been previously identified by Perry et al. (2007) together with species of two other cleistothecial genera *Orbicula* and *Lasiobolidium,* and the apothecial genus *Pseudombrophila*. It should be mentioned, however, that *L. orbicularis* is not the type species of the genus *Lasiobolidium* and the type, *L. spirale,* may be in a different clade. The taxonomic relationships of these and other cleistothecial genera has long been a matter of debate (e.g. Benny and Kimbrough 1980) and molecular data indicate that cleistothecial forms, with loss of active spore discharge, have evolved independently several times within the Pyrenomataceae (Hansen et al. 2005; Perry et al. 2007). With Ascodesmidaceae nested within the Pyronemataceae and several pyronemataceous taxa resolved outside the family, Pyronemataceae as currently circumscribed is not monophyletic (Perry et al. 2007).

Heydenia arietina was described as a species of *Onygena* (Fischer 1897) because it was growing on the horn of an old ram and was superficially somewhat similar to *Onygena equina* and *O. corvina*, both keratinophilic. The placement in *Onygena* was questioned repeatedly by the author himself (Fischer 1897, 1920), but it is surprising that he did not see the close morphological similarities with *H. alpina* and *H. americana* Sacc. & Ellis (Saccardo 1882). This taxonomic misjudgement prompted his contemporary mycologist Rytz (working at the same Botanical Institute as Fischer in Bern, Switzerland) to transfer also *H. alpina* to the genus *Onygena* (Rytz 1923), but this proposal was not followed by other mycologists. Our results clearly show that the two genera are not closely related (Fig. 2).

Morphological comparison of the fruiting bodies of *Orbicula* and *Heydenia*

The close molecular affinity of the two stipitate *Heydenia* species with the sessile *Orbicula parietina* is confirmed by the morphology of their cleistothecia, already described by Hughes (1951). In both genera, the peridium of the cleistothecium consists of two layers. The outer layer is composed of puzzle-like cells with brown, thick walls; the inner layer is made from thin-walled, pale brown to almost colourless polyhedral cells.

According to Hughes (1951), Udagawa and Furuya (1972) and Campbell et al. (1991), *O. parietina* produces fine, thin-walled, evanescent, colourless hyphae (called paraphyses). Young cleistothecia of *H. arietina* contain similar evanescent hyphae, densely packed below the spore mass and in more isolated positions between the spores.

In *Orbicula* elongate asci with uniseriate ascospores are present in young cleistothecia, but they are evanescent, disintegrate early and are absent from mature fruiting bodies (Hughes 1951). In young cleistothecia of *H. arietina* no entire asci were seen, but uniseriate columns of eight ascospores and wall fragments occasionally associated with them strongly suggest that evanescent, early disintegrating asci are also formed in this genus. As it was not possible to obtain *Heydenia* fruiting bodies in culture, we are not able to document asci with better evidence.

The spores of *Orbicula* and *Heydenia* are regularly ellipsoid to spherical, moderately thick-walled, smooth, colourless and devoid of germ pores. The spore walls of both generea have the same staining properties; they are inamyloid, do not stain in Congo red and cotton blue, but the outer wall layer slowly stains greyish blue in toluidine blue.

Thick-walled, cylindrical, pale or colourless, smooth hyphae originate at the base of the cleistothecium of *Orbicula* and from the basal part of the stipe of *Heydenia*. In both cases the hyphae grow out of the outer layer of the peridium (*Orbicula*) or the stipe wall (*Heydenia*).

The two most important differences of *Orbicula* compared to *Heydenia* are the absence of a stipe and the lack of radiating hyphae in the gleba. A minor difference may concern the behaviour of the spore walls in different mounting media. When mounted in 5 % ammonia the spores of *O. parietina* abruptly expand, increasing their mean diameter from about 12 μm to 14.5 μm and roughly doubling the volume, dramatically thinning the inner wall layer and fracturing the outer one. Fragments of the outer layer may even break loose from the spore. This behaviour was not observed in *Heydenia* spores, although they swell slightly in ammonia.

Heydenia arietina differs from *H. alpina* by bigger spores (7.9-9.8 x 5.2-6.8 μm, N=357; against 4.4-5.7 x 3.3-4.5 μm, N=255), stouter fruiting bodies, a non-triangular coronula, and a cleistothecium wider than high.

The presence of asci in young cleistothecia of *Heydenia*

Although no entire ascus was ever seen in our preparations, we think that the *Heydenia* fruiting body is a teleomorph with early evanescent asci in a stipitate cleistothecium. We base our conclusion on the presence of several radially oriented linear groups of eight spores, the presence of possible ascus wall fragments in the immediate surroundings of some of those linear groups, and the similarity of the *Heydenia* spore capsule with the *Orbicula* cleistothecium.

In longitudinal sections near the axis of the fruiting body, the linear groups of eight spores are about parallel to the cutting plane and were seen in their entire length, and they consisted always of eight spores. In other, more lateral sections, the spore columns were more oblique and, therefore, not present in their entire length, and the number of spores present in the section was lower than eight. It is very unlikely that columns of eight spores are formed by pure chance, and the fact that several such columns were seen in a single spore capsule greatly increases the probability that they stem from asci.

The stout radiating hyphae present in the spore mass of the *Heydenia* cleistothecia are not the spore producing structures contrary to the report by Fresenius (1852) and Heim (1934), but in agreement with Fischer (1920) and Rytz (1923).

In mature *Heydenia* cleistothecia no ascogenous hyphae could be identified with any confidence. Presumably they disintegrate early, as do the asci. The narrow, thin-walled and empty hyphae present in the spaces between the moniliform parts of the radiating hyphae (Fig. 5a, b) may be part of the ascogenous system; and some of the loose wall fragments observed between the spores in squash mounts may stem from those hyphae.

Faulty observations suggested pleurogenous chains of conidia

If the radiating hyphae are not spore producing structures, how did Fresenius (1852) arrive at his conclusion that the spores are formed in pleurogenous chains? First, his microscope was not based on optical calculations but was built by empirical trial and error methods (the first microscope based on optical calculations was produced in 1872 by Zeiss). Most important, it lacked an Abbe condenser and an oil immersion lens, as these became commercially available long after his work on *Heydenia* (Abbe condenser 1869, oil immersion 1878). Therefore the resolving power of his instrument was low compared with today's microscopes, and the optical effect created by overlapping Becke lines (Bass et al. 2010) of two adjacent spores was very pronounced, resulting in a small bright spot between the two spores that Fresenius may have taken for a minute connecting cell. Even modern microscopes produce Becke lines and a bright spot between spores in close contact (Fig. 5g), and the intensity of the phenomenon can be increased by closing the aperture diaphragm of the condenser, thus approaching the insufficient optical conditions of Fresenius' microscope. Second, his microscope was insufficient to clearly see the true nature of the small wart-like incrustations on the radiating hyphae (Fig. 4c), and so he mistook them for spore producing wall structures. The H-connections between two radiating hyphae frequently break in squash mounts, leaving conical stumps with a flat open top (Fig. 4c) that may resemble structures producing conidia. Fresenius was then reinforced in his conclusion by the fact that no clearly visible asci were present, and the idea of early evanescent asci may not have occurred to him.

Conclusions

Asci of *Heydenia* appear to disintegrate at an early stage and no entire ascus was seen, but the presence of ascus wall fragments and linear groups of eight spores suggest that the *Heydenia* fruiting body is a cleistothecial teleomorph similar to that of *Orbicula*. Despite the close molecular, morphological and ecological relationship of *Heydenia* and *Orbicula* we refrain from uniting the two genera under a single name (that would have to be *Heydenia*), because *Orbicula* lacks the characteristic stipe and radiating hyphae of *Heydenia,* and *Heydenia* lacks the explosive behaviour of the inner wall layer of *Orbicula* spores when placed in alkaline solutions.

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